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METABOLISM OF CREOSOTE BY CERTAIN MARINE MICROORGANISMS

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METABOLISM OF CREOSOTE BY CERTAIN MARINE MICROORGANISMS

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by

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ABSTRACT

Laboratory experiments showed that microorganisms present on creosoted piling in Port Hueneme harbor were found to have the ability to metabolize creosote, causing loss of creosote and producing chemical changes similar to those which occur naturally in creosote exposed in a submerged harbor environment. This metabolism appears to be restricted to the aromatic hydrocarbon components. Two of these, naphthalene and phenanthrene, were shown to be metabolized to a significant extent by certain marine microorganisms.

INTRODUCTION

The destruction by marine boring organisms of wooden structures exposed to the sea has long been a problem and a detriment to economy. Of the many preservative systems used to deter the action of marine borers, the most successful has been pressure impregnation with coal tar creosote. Although creosoted piling may last for thirty or more years in cold water harbors, service lives of from five to ten years are common in warm water harbors.

The manner in which creosoted piling loses resistance to marine borer attack has never been established with any degree of certainty. Hochman et al.[1] found that the outer surface of a creosoted pile that had been exposed in Port Hueneme harbor for approximately fifteen years was honeycombed with Limnoria even though considerable quantities of creosote were present. When the creosote was extracted from this honeycombed outer layer and tested for toxicity according to the procedure of Vind and Hochman,[2] it was found to be relatively nontoxic to Limnoria. Creosote extracted from deeper portions of the pile was found to be still quite toxic to Limnoria.

Recently, a number of bacteria were reported[3] to be present on the surface of creosoted piling, and one of the most predominant and widespread species, Pseudomonas creosotensis, was described on the basis of standard morphological and biochemical tests. An investigation was conducted to determine if microorganisms present on the surface of marine piling had the ability to metabolize creosote to (1) effect chemical changes in the creosote such as occur in creosoted piling in sea water and (2) reduce the amount of creosote present.

EXPERIMENTAL

Changes in Composition of Creosote in Marine Piling

A creosoted piling was removed from Port Hueneme harbor after seventeen years of service, and the portion constituting the intertidal zone was brought to the laboratory. The honeycombed outer shell (Layer #1) was removed by scraping with a steel spatula and wire brush. The wood immediately below the honeycombed shell (Layer #2) was removed by shaving with a plane to a depth of 1/8 inch. Finally, a second 1/8 inch of piling (Layer #3) was removed with a chisel. Each of the three

different portions of fragmented piling was extracted for two days with benzene in a modified Soxhlet extractor. After removal of the benzene from the extracts by distillation under reduced pressure, each creosote residue was analyzed for saturated hydrocarbon content by the method of Drisko and Hochman,[4] tested for toxicity to Limnoria by the method of Vind and Hochman,[2] and analyzed on a Beckman IR-5 spectrophotometer to detect chemical differences.

Loss of Creosote in the Presence of Marine Microorganisms as Determined by Weighing

Whole Creosote. To each of sixteen 250-ml Erlenmeyer flasks containing 50.00 ml of autoclaved sea water nutrient broth (0.8 percent by weight of powdered broth) was added, from a micropipette, approximately 250 mg of creosote. A tared flask and micropipette were used so that the weight of added creosote could be determined by difference to the nearest milligram. A creosoted piling splinter that had been aseptically removed from below the water line in Port Hueneme harbor less than one-half hour previously was added to each of half of these flasks. A creosoted piling splinter that had been autoclaved in sea water was added to each of the remaining flasks. All flasks, which had been fitted with cotton plugs and handled under sterile conditions, were incubated in an Eberbach rotary shaker maintained at 74 F ± 2.

Periodically, pairs of flasks (one inoculated and one control) were randomly selected for analysis as follows: The splinter was removed and the contents of each flask were quantitatively transferred to a reverse phase liquid-liquid extractor and extracted with carbon tetrachloride. Extraction was continued for six hours. The carbon tetrachloride solution was then dried over sodium sulfate, filtered, and evaporated to dryness under reduced pressure. The residue was weighed to the nearest milligram and an infrared spectrum was recorded.

An agar plate was poured with a loopful of broth from each flask 24 hours after inoculation to insure that each inoculated flask had living organisms and each control flask was sterile. This was also done routinely with all flask contents in later experiments on weight losses of creosotes and creosote constituents.

Chromatographed Creosote. The method used was that described for whole creosote except that cork stoppers wrapped in metal foil were used in place of cotton plugs in order to reduce losses due to volatilization. The chromatographed creosote was creosote that had been placed on a column of silica gel and exhaustively eluted with n-hexane to obtain a mixture of light yellow oil and solids; the mixture was composed almost entirely of aromatic hydrocarbons. For convenience, this chromatographed creosote will be called creosote A.

Loss of Creosote in the Presence of Marine Microorganisms as Determined by Spectrophotometry

250-mg Samples of Whole Creosote. One set of twenty-six 250-ml Erlenmeyer flasks containing 50.00 ml of sea water nutrient broth (0.8 percent by weight of powdered broth) and a second set of six 250-ml Erlenmeyer flasks containing 50.00 ml of sea water nutrient broth (0.08 percent by weight of powdered broth) were sterilized by autoclaving. To each of the 32 flasks was added approximately 250 mg of creosote weighed by difference to the nearest milligram. Half of each set of flasks were inoculated with creosoted piling splinters containing bacteria, and half with sterile splinters, as previously described. Seven pairs of flasks of 0.8 percent broth were incubated in a reciprocating shaker at 100 F ± 2, and six pairs of flasks of 0.8 percent broth and three pairs of flasks of 0.08 percent broth were incubated in a rotary shaker at 62 F ± 2.

To determine the amount of creosote present in each flask, the spectrophotometric method of Kolb, Hribar and Streed[5] was used. This method utilizes the absorbance of a solution of creosote in chloroform at 254 mμ as a measure of concentration. A calibration curve was prepared in which absorbance was plotted against concentration of creosote.

Periodically pairs of flasks (one inoculated and one control) were randomly selected for creosote analysis. The splinter was removed from the flask, and the contents were quantitatively transferred into a separatory funnel. The creosote was then extracted into 50.00 ml of chloroform. This solution was diluted 1 : 1,000 with chloroform, and the absorbance was measured on a Beckman DU spectrophotometer at 254 mμ. The absorbance reading was converted to concentration by use of the calibration curve, and the actual loss of weight of creosote was recorded.

Creosote Samples Larger Than 250 mg. Six 250-ml Erlenmeyer flasks containing 50.00 ml of sea water broth (0.8 percent by weight of powdered broth) were sterilized by autoclaving. To each of one pair of flasks was added from a micropipette approximately 1.0 g of creosote, to each of another pair of flasks was added approximately 1.5 g creosote; and to each of a third pair of flasks was added approximately 2.0 g of creosote. The creosote was weighed by difference to the nearest milligram. One of each of the three pairs of flasks was inoculated with 1 ml of a mixed culture of microorganisms that had been growing in a suspension of 4 percent creosote in nutrient broth. All six flasks were stoppered with corks covered with metal foil and incubated in a reciprocating shaker maintained at 62 F ± 2. After four weeks all flasks were removed and analyzed for creosote content by the same method used for the 250-mg samples.

Chromatographed Creosote. Two creosote chromatography fractions were used. One was creosote A which comprised about 77 percent of the whole creosote. The other was that portion of the whole creosote that remained on the silica gel column during elution of creosote A with n-hexane but was eluted with methanol. For convenience, the methanol-eluted fraction will be called creosote B. This comprised about 21 percent of the total creosote. A pair of flasks containing creosote A and two pairs of flasks containing creosote B were treated in the same way as the 250-mg samples of whole creosote. Spectrophotometric measurements of absorbance were made at the wavelengths of their maximum absorption, 254 mμ for creosote A and 254.5 mμ for creosote B. The absorbances were then converted into concentrations by use of reference concentration curves prepared for each of the two chromatography fractions. It should be noted that creosotes A and B had nearly the same absorbance at 254 mμ, showing that the spectrographic analysis of the 250-mg samples of whole creosote was not determined by only one class of creosote constituents.

Metabolism of Compounds in Creosote in the Presence of Marine Microorganisns

Approximately 250 mg of naphthalene, acenaphthene, anthracene, phenanthrene, and n-octadecane, five compounds known to be present in creosote, were each added to individual 250-ml Erlenmeyer flasks containing 50.00 ml of autoclaved sea water nutrient broth (0.8 percent by weight of powdered broth) and weighed by difference to the nearest milligram. Part of the flasks were inoculated with splinters of creosoted piling recently removed from Port Hueneme harbor, part were inoculated with 1 ml of a 48-hour culture of microorganisms from creosoted chips in sea water nutrient broth, and part were kept for sterile controls. All inoculated and sterile control flasks were fitted with metal-foil-covered stoppers and incubated in an Eberbach rotary shaker maintained at 68 F ± 2. Periodically, pairs of flasks (one inoculated and one control) were randomly selected and analyzed for the amount of creosote constituent present by direct weighing or by spectrophotometry.

Determined by Weighing. It was necessary to determine the amount of n-octadecane present in the flasks by direct weighing since n-octadecane does not have a strong absorption peak in the ultraviolet region of the spectrum. It was also convenient to analyze for phenanthrene by the liquid-liquid extraction procedure previously described for creosote, because it is not lost by volatilization to any significant extent during the evaporation of the carbon tetrachloride solution.

Determined by Spectrophotometry. Calibration curves were prepared for naphthalene, acenaphthene, anthracene, and phenanthrene in chloroform solution at 277.5 mμ, 293 mμ, 255.5 mμ, 251 mμ, respectively, so that the concentration of each of the creosote components could be determined from the absorbance of the solution. The weights of these compounds were determined spectrophotometrically at the above wavelengths and at appropriate dilutions by the method previously described for creosote.

RESULTS

Changes in Composition of Creosote in Marine Piling

In Table I is recorded the percent of creosote in each piling layer, the toxicity of the creosote and percent of saturated hydrocarbons in each of the three creosotes extracted. There were two major differences in the infrared spectra of the creosotes extracted from the outer and the two inner layers. The spectrum of the outer creosote had a much greater absorption peak near 3.4 μ as compared to the absorption peak near 3.3 μ (indicating a greater proportion of methylene groups) and an absorption peak at 5.85 μ (indicating the presence of carbonyl groups, which did not occur in the spectra of the other two extracts). Also, a small but sharp absorption peak occurred near 9.9 μ in the spectrum of the creosote from Layer #3 which did not occur in the other two spectra. The spectra of these creosote extracts are discussed more fully in TR-198.[6]

Table I. Properties of Creosotes from Different Cross-Sectional Piling Depths

Layer	Creosote in Piling Layer (% by weight)	Concentration to Kill 50% Limnoria (ppm)	Saturated Hydrocarbons (% by weight)
#1*	11.2	77	4.5
#2	25.6	10	2.1
#3	24.2	15	2.0

* Outer

Loss of Creosote in the Presence of Marine Microorganisms as Determined by Weighing

Whole Creosote. All agar plates poured with broth from control flasks contained no growth, and all agar plates poured with broth from inoculated flasks contained living organisms. This was true for all experiments on weight losses of creosotes and creosote constituents.

The loss of weight of creosote from each flask is shown in Table II. Creosote losses from inoculated flasks were consistently higher than those from the corresponding control flasks. The high creosote losses from the control flasks were attributed to

vaporization of the more volatile components of creosote. A statistical analysis of weight losses is presented in Appendix A. It indicates that the creosote losses from inoculated flasks were significantly greater than those from control flasks; reliability is 95 percent.

Table II. Loss of Weight of Creosote

Incubation (days)	Loss from Inoculated Flask (mg)	Loss from Control Flask (mg)	Difference in Losses (mg)
0	39	35	4
1	36	37	-1
4	161	110	51
7	199	108	91
9	195	106	89
11	171	163	8
14	181	101	80
16	153	151	2

Compared with creosotes extracted from control flasks those extracted from inoculated flasks had a consistently greater absorbance peak near 3.4 μ and smaller absorbance peak near 3.3 μ. The spectra of the former creosotes also had a greater absorbance at 5.9 μ. A small but sharp absorption peak near 9.9 μ occurred in the spectra of all creosotes from control flasks, but this peak was either diminished or completely lost in the spectra of creosote from inoculated flasks. Spectral differences were greatest when the inoculated creosotes had the greatest weight losses. These spectral differences occurred to all creosote pairs examined by weighing.

Loss Determined from Chromatographed Creosote

The loss of weight of creosote from each flask is shown in Table III. Again creosote losses from inoculated flasks were consistently greater than those from the corresponding control flasks. Weight losses in control flasks were greatly diminished for the first twelve days as compared to the preceding experiment, but the fourteen- and sixteen-day control flasks had large losses. A statistical analysis of weight

losses by the method in Appendix A indicates that the creosote losses from the inoculated flasks were significantly greater than those from the control flasks; the reliability is 99.5 percent.

Table III. Loss of Weight of Creosote A

Incubation (days)	Loss from Inoculated Flask (mg)	Loss from Control Flask (mg)	Difference in Losses (mg)
2	87	21	65
4	156	36	120
6	193	40	153
8	120	26	94
10	159	45	114
12	218	42	176
14	198	117	81
16	179	130	49

Loss Determined by Spectrophotometry for Whole Creosote

250-mg Creosote Samples. The loss of weight of creosote from each flask is shown in Table IV. Creosote losses from inoculated flasks were in all cases greater than those from corresponding control flasks. The mean loss for inoculated flasks at 62 F was greater than that for inoculated flasks at 100 F, even though the biological growth was greater at the higher temperature. Creosote losses did not steadily increase with time but fluctuated about the mean value. The smaller creosote losses with 0.08 percent broth were probably due to a smaller bacterial population.

The ultraviolet spectrophotometric method of analysis was effective in minimizing the loss of creosote from control flasks. The greater losses from control flasks at 100 F are attributed to increased volatilization at the higher temperature.

When weight losses from all sixteen pairs of flasks were combined, a statistical analyses by the method in Appendix A indicated that the creosote losses from inoculated flasks were significantly greater than those from corresponding control flasks; the reliability is 99.5 percent.

Table IV. Change in Weight of Creosote

Incubation (days)	0.8% Broth at 100 F		0.8% Broth at 62 F		0.08% Broth at 62 F	
	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)
3	-146	-6	-139	+16	-35	+1
4	-128	-60	-152	-20	—	—
5	-85	-53	-164	+1	—	—
6	—	—	—	—	-103	+5
7	-81	-60	-148	-12	—	—
9	-97	-43	-120	-24	-82	+2
11	-111	-85	-167	-30	—	—
13	-136	-38	—	—	—	—
Mean Loss	112	49	148	12	73	3 Gain

Creosote Samples Larger Than 250 mg. The loss of weight of creosote from each flask is shown in Table V.

Table V. Change in Weight of Creosote

Sample Number	Bacterial Treatment	Original Weight of Creosote (g)	Final Weight of Creosote (g)	Change in Weight of Creosote (mg)
1	control	1.040	1.080	+40
2	inoculated	1.007	.630	-377
3	control	1.499	1.500	+1
4	inoculated	1.486	1.375	-111
5	control	2.010	2.000	-10
6	inoculated	1.986	1.800	-186

Loss From Chromatographed Creosote

The weight losses of creosote A and B are given in Table VI. From Tables III and VI it appears that creosote A was metabolized to a significant extent by marine microorganisms but that creosote B was not.

Table VI. Change in Weight of Creosotes A and B

Sample	Incubation (days)	Change of Weight (mg)
Creosote A, inoculated	8	-125
Creosote A, control	8	+2
Creosote B, inoculated	9	-25
Creosote B, control	9	-25
Creosote B, inoculated	17	-22
Creosote B, control	17	-15

Determined by Weighing. The loss of weight of n-octadecane from each flask is given in Table VII.

Table VII. Change in Weight of n-Octadecane

Incubation* (days)	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)
5	+2	0
6	-17	-10
8	+24	+14
12	0	+4

* Splinters of creosoted piling were used to inoculate flasks.

The loss of weight of phenanthrene from each flask is shown in Table VIII.

Table VIII. Change in Weight of Phenanthrene

Incubation* (days)	Loss From Inoculated Flask (mg)	Loss From Control Flask (mg)	Difference in Losses (mg)
3	20	10	10
5	40	3	37
7	107	3	104
10	90	12	78
12	122	5	117
14	170	6	164
17	119	4	115

* Splinters of creosoted piling were used to inoculate flasks.

Weight losses from inoculated flasks were consistently greater than those from corresponding control flasks. A statistical analysis of the weight losses by the method in Appendix A indicates that losses from inoculated flasks were significantly greater than those from corresponding control flasks; reliability is 99.5 percent.

Determined by Spectrophotometry. Weight losses of naphthalene, acenaphthene, anthracene, and phenanthrene are given in Tables IX through XII.

Table IX. Change in Weight of Naphthalene

Incubation (days)	Inoculated With	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)
6	splinter	-168	+1
8	splinter	-171	-1
9	splinter	-139	-11
11	1 ml broth	-104	+3
12	splinter	-233	-6
12	1 ml broth	-204	-6
18	1 ml broth	-138	-3
26	1 ml broth	-243	+7

Table X. Change in Weight of Acenaphthene

Incubation (days)	Inoculated With	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)
6	splinter	-39	-6
8	splinter	+2	-7
9	splinter	-25	+10
10	1 ml broth	+1	*

* Above control used

Table XI. Change in Weight of Anthracene

Incubation (days)	Inoculated With	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)
6	splinter	+1	+5
8	splinter	+49	+12
9	splinter	+16	+1
10	1 ml broth	+48	*

* Above control used

Table XII. Change in Weight of Phenanthrene

Incubation (days)	Inoculated With	Weight Change in Inoculated Flask (mg)	Weight Change in Control Flask (mg)
6	splinter	-55	+3
8	splinter	-68	+2
9	splinter	-69	+2
10	1 ml broth	-26	*
12	splinter	-100	+3
28	splinter	-223	+12

*Above control used

DISCUSSION

The analysis of creosote extracted from different piling depths showed that a significant chemical change had occurred in the creosote from the outer portion of the pile, as well as a significant loss of weight of creosote. There was a greater percent of saturated hydrocarbons and a greater percent of carbonyl-containing compounds in the outer creosote than in the creosote further in the interior of the

piling. The former change may have been due in large part to differential leaching, but the latter was undoubtedly due to oxidation of the creosote, known to occur during field service.[8] The chemical change in the outer creosote was accompanied by a considerable reduction in toxicity to Limnoria.

All experiments conducted to determine the loss of creosote in the presence of marine microorganisms indicated that creosote losses were greater in the presence of these organisms than in sterile control flasks. In analyses by direct weighing, control samples of creosote showed significant weight losses from evaporation of carbon tetrachloride solutions, but weight losses by vaporization were considerably diminished in spectrophotometric analyses. The spectral changes in the weight-loss experiments were similar to changes that occurred to creosote in the outer portion of the Port Hueneme pile, namely an increase in the relative amounts of methylene groups and oxidation products. Thus, some of the creosote components had undergone a change in chemical composition similar to that which occurred to the creosote in the piling.

Experiments conducted on chromatography fractions indicate that creosote losses in the presence of marine microorganisms are restricted to the aromatic hydrocarbon portion.

From the experiments conducted on compounds in creosote, it was determined that naphthalene and phenanthrene were metabolized by marine microorganisms present on the surface of creosoted piling in Port Hueneme harbor but that acenaphthene, anthracene, and n-octadecane are not metabolized to any appreciable extent. The greater rate of metabolism of naphthalene over phenanthrene may be due to the greater solubility of naphthalene. The bacterial oxidation of naphthalene to salicylic acid has been reported by Klausmeier and Strawinski.[9]

FINDINGS

1. Distinct chemical differences were found between creosote extracted from the outer shell of a seventeen-year-old Port Hueneme marine pile and that extracted from the next two adjacent layers of this pile.

2. These chemical differences include an increase in the percent of saturated hydrocarbons and oxidation products.

3. The toxicity of the outer creosote was significantly less than that of the creosote extracted from the two adjacent inner portions of the pile.

4. Creosote losses from nutrient broth inoculated with creosoted piling splinters containing marine microorganisms were greater than those from corresponding sterile control flasks.

5. The greater creosote losses from inoculated flasks were shown both by direct weighing and by spectrophotometry.

6. Infrared study of creosote exposed to marine microorganisms showed that chemical changes had occurred similar to those which occurred to creosote in the outer portion of the seventeen-year-old marine pile.

7. Creosote losses appear to be restricted to the aromatic hydrocarbon fraction.

8. Naphthalene and phenanthrene were metabolized by marine microorganisms, but acenaphthene, anthracene, and n-octadecane were not metabolized to any appreciable extent.

CONCLUSIONS

1. Microorganisms present on creosoted marine piling have the ability to metabolize creosote, to reduce the amount of creosote present, and to alter its chemical composition.

2. Aromatic hydrocarbon components of creosote are the primary substrate for these organisms.

3. Microbial metabolism of creosote may be a significant factor in the accelerated deterioration of pressure-treated creosoted timbers in some harbors.

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Appendix A

ANALYSIS OF WEIGHT LOSSES OF WHOLE CREOSOTE

NULL HYPOTHESIS: The weight losses are the same, H_o : i.e., $L_1 = L_2$ or $\Delta L = X = 0$

$$n = 8$$

$$T = \Sigma X = 324$$

$$\bar{X} = \frac{\Sigma X}{n} = 40.5$$

$$\Sigma X^2 = 25{,}288$$

$$S_X^2 = \frac{1}{n-1}\left(\Sigma X^2 - \frac{T^2}{n}\right) = 1{,}738$$

$$S_{\bar{X}}^2 = \frac{S_X^2}{n} = 217.3$$

$$S_{\bar{X}} = 14.74$$

TEST STATISTIC

$t = [(\bar{X} - 0)/S_{\bar{X}}] > t_\alpha\,(n-1) = t_{.05}(7)$ where $t_\alpha\,(n-1)$ is Student's t distribution with $n - 1$ degrees of freedom at the α level of significance.

$$t_{.05}(7) = 2.36$$

$$t = 2.75 > 2.36$$

Thus, it can be stated with 95 percent reliability that creosote losses from inoculated flasks were significantly greater than those from corresponding control flasks.

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